FURTHER ANALYSIS OF THE PROTECTIVE VALUE OF BIOLOGICALLY CONDITIONED FRESH WATER FOR THE MARINE TURBELLARIAN, PROCERODES WHEATLANDI. IV. THE EFFECT OF CALCIUM

R. B. OESTING AND W. C. ALLEE

(*From the Marine Biological Laboratory and the University of Chicago*)

In a series of studies (1928, 1929, 1933) Allee has shown that the marine turbellarian, *Procerodes wheatlandi,* resists cytolysis longer in extremely hypotonic water, other conditions being equal, if such water has been biologically conditioned than if it lacks biological contamination. These relations hold even though the total concentration of electrolytes, as measured by the conductivity method, is the same. The balance of electrolytes is necessary in such experimental tests, since an increase in their concentration in the proportions found in sea water likewise confers protection.

Protective biological conditioning results when other *Procerodes* have previously been exposed to the fresh water, and especially so if some few *Procerodes* have recently died and disintegrated in it. The protective action of this biologically conditioned fresh water is maintained after dialysis to the same conductance as that of extremely dilute sea water controls. Water extracts of marine amphipods and of fresh water planarians, *Euplanaria novangliæ* (=*Planaria maculata*), show these same effects, as does water from cultures of the latter and water from sterilized hay infusions of a practically pure culture of *Paramecium.* The protective effects are not due to pH differences, or to a depressing action such as is produced by exposure to dilute alcohol or to ethyl urethane. The protective agent is not adsorbed by activated charcoal, or by coagulated egg white.

The onset of cytolysis was determined in these experiments by inspection with a hand lens. The results have been confirmed by histological studies on worms taken from Allee's 1933 experiments (Fowler, 1935).

Pantin (1931*a* and *b*) and Weil and Pantin (1931) have studied the adaptation of *Procerodes* (=*Gunda*) *ulvæ* to fresh waters. Their results bear on our problem since the two species are closely related taxonomically and in habitat toleration. *P. ulvæ* invades small streams where conditions are suitable; it may extend up these to, or slightly above, mean high water at neap tides (Ritchie, 1934). The reaction of this planarian in fresh waters varies with the chemical composition of the water. The worms swell less and recover more completely on their return to sea water if placed in fresh waters rich in calcium rather than in calcium-deficient waters. In nature, Ritchie (1934) has found them living in fresh water with as little as 5 mgm. per liter of calcium for as long as five days when periods of calm weather, with the resulting lack of salt splash, coincide with neap tides.

Materials and Methods

P. wheatlandi were collected daily from the same locality as in the previous studies of Allee. *Euplanaria novangliæ* were collected from a fresh water pond known to be low in salt content; the pond water used came from the same pond which was also the source of these planarians and of pond water in Allee's latest work (1933).

The interval before the beginning of cytolysis in hypotonic waters depends on several factors, among which are the following: Small worms begin and undergo cytolysis more rapidly than do larger ones; worms long in the laboratory are less resistant than are those freshly collected; the more often the medium is renewed, the sooner cytolysis begins. The higher the temperature, or the lower the spcific conductance of the water, the less the resistance of *Procerodes* (cf. Allee, 1933).

Assays of the protective value of various media were made, keeping these factors as nearly identical as possible for the worms whose resistance was being comparatively tested except that variations in specific conductance were used as a tool in the analyses. The worms were first isolated in a small drop of sea water on the curved bottom of the salt cellar type of watch glass; they were then washed five times with the respective media into which they were to be placed. The worms were paired for comparative assays before the test began; the members of each pair were selected for similarity in size, activity, and laboratory age. After the washings, 2 cc. of the respective media were run over each worm; this liquid was renewed every one, two, or four hours, depending on the severity of the media and the laboratory age of the worms. The survivals were all tested at room temperature in a room with north exposure which was not subjected to rapid changes in temperature. The highest temperature recorded for the summer in this room was 25.5° C. Examinations were made every fifteen minutes with a ten-power hand lens until the beginning of fatal cytolysis was noted. Ten worms were isolated into each type of media being tested in an assay.

Conductivity measurements were made using a No. 7651 Leeds and Northrup potentiometer and a Washburn type conductivity cell designed for solutions with low conductance. Specific conductances are given in mhos $\times 10^5$. Allee has previously given conductivity measurements in terms of ohms resistance. His data may be compared with those given in the present report by the use of the following data: under the conditions given, 6,000 ohms $= 5.42 \times 10^{-5}$ mhos; 2,500 ohms $= 13.05 \times 10^{-5}$ mhos and 1,700 ohms $= 19.20 \times 10^{-5}$ mhos.

Calcium analyses were made using the Van Slyke and Sendroy (1929) method. The accuracy of this method to within the one per cent claimed for it was confirmed by tests on the recovery of calcium added to distilled water and to the other media used in this work.

Table I

The Protective Action of Calcium for Procerodes Isolated into Extremely Hypotonic Water

Experiment No.	Medium	Calcium added		Calcium not added		Difference in hours
		Mhos $\times 10^5$	Hours survived	Hours survived	Mhos $\times 10^5$	
5	Tap	8.38	2.74	1.80	7.30	0.94
6	Sea	30.12	2.23	1.25	28.69	0.98
6*a*	Tap	8.83	3.95	2.43	7.15	1.52
8	Tap	21.29	9.33	4.00	7.07	5.33
8*a*	Sea	40.80	9.31	4.03	26.53	5.28
9	Pond	5.02	7.20	3.30	4.79	3.90
10	Pond	5.50	5.50	3.55	4.79	1.95
10*a*	Pond	6.27	15.80	5.32	4.56	10.48
11	Pond	7.43	3.23	2.14	4.79	1.09
12	Pond	7.13	2.63	2.20	4.79	0.43
13	Pond	5.40	4.34	3.00	4.79	1.34
21	Pond	6.25	5.98	4.08	4.79	1.90
Averages			6.02	3.09		2.92

Statistical probability 0.005

Experimental Results

Calcium, as $CaCl_2$, was added to tap, pond and to extremely hypotonic sea water in a group of twelve experiments with results which are summarized in Table I. In each assay, the worms survived longer, on the average, in the water to which calcium had been added than did control worms in otherwise similar water. The amount of calcium used differed in different tests; in all cases the increase in specific conductance is a measure of the calcium added. Further comparisons can be made from the fact that in Experiment 8, 1 cc. of M/10 $CaCl_2$ was added to 200 cc. of tap water and in Experiment 8*a* the same amount was added to 200 cc. of 1 : 300 sea water.

Although varying amounts of calcium were added and further variations were caused by differences in laboratory age and in the number of changes of the media during the assays, when the data given in Table I are considered as twelve paired experiments, the difference in survival of 2.92 hours has a statistical probability of 0.005.[1] The coefficient of correlation between the differences in the amount of calcium as measured by specific conductance and the differences in the resistance of the worms is 0.3691 ; this is statistically significant.

Table II

Relative survival of Procerodes isolated in tap water and in dilute sea water. Time is given in hours and each value is the average for 10 worms; specific conductance is in mhos.

Experiment No.	Dilute sea water		Tap water	
	Survival	Sp. cond. $\times 10^5$	Survival	Sp. cond. $\times 10^5$
1	4.825	19.07	5.725	6.64
2	6.667	20.14	8.566	6.38
3	4.697	25.90	7.865	6.79
4	2.768	28.69	2.100	7.82
5	1.535	27.95	1.800	7.30
6	1.250	28.69	2.425	7.15
7	7.875	39.75	8.150	7.07
8	4.775	26.53	4.000	7.07
Averages	4.299	27.09	5.079	7.03

The relatively greater protection from hypotonic water furnished by calcium in comparison with that given by other electrolytes in the proportions found in sea water is illustrated by the data summarized in Experiments 8 and 8*a*, Table I, and the further data given in Table II. In Experiments 8 and 8*a*, despite the differences in conductance, there is no significant difference in survival of worms in tap water or in 1 : 300 sea water and again there is no significant difference in survival in these two waters after the same amount of calcium is added. There is a decidedly significant difference between the survival of the worms in either water before and after the addition of calcium.

The waterworks of Falmouth, which supplies the tap water used in these experiments, now uses a lime treatment which materially increases the calcium content. This treatment was started since Allee's last pre-

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[1] Values of 0.05 or less are usually considered statistically significant. "Student's" method of paired comparisons was used in all statistical analyses.

ceding experiments. The presence of this calcium probably accounts for the markedly greater protection of *Procerodes* in this water over that which would be expected from its low specific conductance. The dilutions of sea water contained approximately four times as much electrolytes as did the tap water; rough calculations of the calcium content from analyses furnished by the Falmouth waterworks showed this to be practically the same in the two. The survival of the *Procerodes* isolated in the two types of water was approximately the same in both; the mean difference of 0.78 hours longer survival in tap water lacks statistical significance (P. = 0.133).

TABLE III

Survival of Procerodes in planarian-conditioned fresh water as compared with that shown in different control media. Ten worms were assayed in each experiment except No. 13 in which eight were used.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
9	4.025	3.300	7.900	—
10	3.925	3.550	4.650	—
11	3.650	2.650	3.725	2.150
12	2.525	2.275	2.725	2.200
13	4.156	2.594	4.219	3.188
20	1.525	0.750	1.350	1.225
21	5.250	2.925	5.975	4.075
Means	3.579	2.578	4.363	2.568

Statistical analysis

	Difference		Probability	Cases
I–II	0.9900		0.0000	68
III–I	0.9375		0.0004	68
III–II	1.7941		0.0000	68
I–IV	0.8490		0.0010	48
IV–II	0.3125		0.2508	48
III–IV	1.0313		0.0000	48

It is evident from these data that calcium delays the onset of cytolysis when *Procerodes* are isolated in extremely hypotonic fresh waters. Any factor which would tend to increase the amount of calcium present then, should confer such protection. This leads to the fundamental question of the present inquiry: Is the protective value of biologically conditioned water to be attributed to a differential increase in calcium which accompanies conditioning? It is at once obvious that the addition of sea water to a control sample of fresh water until its total electrolyte content equals that of a biologically conditioned sample as measured by the conductivity method, while furnishing a basis for an adequate check on protection due to increased osmotic pressure, gives inadequate control over any specific electrolyte such as calcium. It is necessary to test directly for the amount of calcium present and to confirm its effectiveness in biologically conditioned water.

Unless otherwise stated, the amount of biological conditioning used was that furnished by 200 *Euplanaria novangliæ* living in one liter of fresh pond water for approximately 44 hours, or its equivalent obtained by using fewer worms for a longer period. Sea water and calcium chloride were added respectively to other portions of fresh pond water until the specific conductance of all three solutions was the same. As an additional control, except in Experiments 9 and 10, a sample of untreated pond water was assayed together with the above media. The average survival times of *Procerodes* from seven such experiments are shown in Table III.

TABLE IV

Calcium analyses of assayed samples of media from Table III; results shown in mgm. calcium per liter.

	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
Calcium content	2.072	0.809	3.410	1.018

These data, together with the statistical analyses, show that planarian-conditioned water has a protective value lying between that of pond water plus sea water and pond water plus calcium chloride when all are of the same electrolytic content. When, however, the amount of calcium present in the planarian culture water (Table IV) was determined and calcium chloride was added to pond water to bring the calcium content to that of the planarian-conditioned water, no difference could be detected between the effectiveness of these two waters in protecting the *Procerodes* isolated in them. These results are summarized, together with a statistical analysis, in Table V.

To determine whether any factor other than calcium might be involved in the protection furnished by planarian-conditioning, a medium

TABLE V

Procerodes survival in pond water and planarian-conditioned pond water with the same calcium content.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + sea water	IV Pond water + $CaCl_2$
26	5.250	5.650	3.850	8.300
27	1.875	2.150	1.150	3.500
Means	3.563	3.900	2.500	5.900

Statistical analysis

	Difference	Probability	Cases
I– II	−0.3375	1.0000	20
I–III	1.0625	0.0396	20
IV– I	2.3375	0.0052	20
II–III	1.4000	0.0230	20
IV– II	2.0000	0.0344	20
IV–III	3.4000	0.0000	20

was made up to represent a synthetic river water (Henderson, 1913, p. 113). To each liter of water the following salts were added:

100 mgm. $CaCl_2$
50 mgm. $MgSO_4$
25 mgm. NaCl
10 mgm. KCl

This water in full, half- and quarter-strengths, when conditioned by planarians, showed no change in the total electrolytic content; likewise there was no protection for *Procerodes* when compared by the usual assay to the survival shown in similarly treated but unconditioned synthetic river water. The results of these experiments together with a statistical analysis are summarized in Table VI. From all this evidence, we can safely conclude that an increase in calcium is the factor furnishing the protection to *Procerodes* in planarian-conditioned pond water.

Allee's previous work had shown that water extracts of *Procerodes* themselves furnished a potent protection for other worms of the same species isolated in extremely hypotonic media. The relation between this protection and the calcium content of the water was also tested.

TABLE VI

A summary of tests which indicate that the protection furnished by calcium is sufficient to account for the protective value of planarian-conditioned water. Each of the survival times given is the average for ten worms.

Experiment number	Hours survival		Amount of conditioning	Laboratory age of *Procerodes*	Mhos × 10^5	Media strength	Media changed	Difference
	Planaria culture	Control						
14	10.225	5.625	100 worms in 250 cc. 16 hrs.	1 day	48.70	Full	Every hour	+4.60
15	6.550	6.800	100 worms in 500 cc. 44 hrs.	2 days	48.70	Full	Every hour	−0.25
16	21.75	19.30	14 worms in 250 cc. 68 hrs.	over 5 days	48.70	Full	Every hour*	+2.45
17	5.65	6.30	200 worms in 500 cc. 20 hrs.	3 days	26.15	Half	Every 2 hours	−0.65
18	6.95	7.15	100 worms in 500 cc. 44 hrs.	4 days	26.15	Half	Every 2 hours	−0.20
19	7.00	6.20	60 worms in 500 cc. 68 hrs.	4 days	26.15	Half	Every 2 hours	+0.80
23	5.50	6.90	200 worms in 500 cc. 20 hrs.	1 day	12.32	Quarter	Every 2 hours	−1.90
24	8.90	9.35	100 worms in 500 cc. 44 hrs.	2 days	12.32	Quarter	Every 2 hours	−0.45

Average .... +0.55
Statistical Probability 0.4500

* Media exhausted after six changes.

Procerodes extract was prepared as in the earlier work. After five washings with distilled water, 500 *Procerodes* were boiled in distilled water for about 15 minutes. The beaker was then covered and set aside for 24 hours. Controls were made up to the same specific conductance as the extract by adding calcium chloride to distilled water and sea water to distilled water respectively. All three media were then assayed for their protective value to *Procerodes* and were then analyzed for calcium. It is evident from an examination of Tables VII and VIII which summarize these data that calcium is also the factor in *Procerodes* extracts which furnishes the observed protection for *Procerodes*. Since making a water extract of *Procerodes* is a short-cut method for preparing *Procerodes*-conditioned water, it is unnecessary to examine this possibility for further confirmatory evidence that an increase in calcium is the fac-

TABLE VII

The protective value of *Procerodes* extracts for *Procerodes* isolated in hypotonic media; each survival time given is the average for ten worms.

Experiment No.	Hours survival		
	I *Procerodes* extract	II Dist. H_2O + $CaCl_2$	III Dist. H_2O + sea water
28	6.150	6.350	4.150
29	2.100	3.000	1.750
Means	4.125	4.675	2.950
	Statistical analysis		
	Difference	Probability	Cases
I–II	0.550	0.5280	20
I–III	1.175	0.0344	20
II–III	1.725	0.0050	20

TABLE VIII

Calcium analyses of assayed samples from Table VII; results shown in mgm. per liter.

	Procerodes extract	Dist. H_2O + $CaCl_2$	Dist. H_2O + sea water
Calcium content	7.20	8.66	0.42

tor furnishing protection to *Procerodes* in biologically conditioned fresh waters.

Discussion

These experiments were carried on by essentially the same methods used by Allee in his preceding studies and support his results wherever similar points were being tested. Specifically, in addition to more general relations, these experiments confirm the earlier findings, that, other conditions being equal, *Procerodes* survive longer (1) if the osmotic pressure is increased by the addition of sea water; (2) if the hypotonic water is biologically conditioned (*a*) by the presence of living fresh water planarians or (*b*) by the presence of water extracts of freshly killed *Procerodes*.

In addition, the earlier results are extended by the demonstration that there is more calcium added than would be expected from its proportionate concentration in sea water and that calcium has protective value greater than would be expected from its osmotic effect. There is nothing in the earlier experiments which is out of harmony with the present findings.

In certain of Allee's work (1929, 1933) the conditioned water was dialyzed to bring the specific conductance to the desired experimental level. Such dialyzed solutions were definitely protective. This protection can be explained adequately in the light of the present work if one considers the amounts of calcium introduced into conditioned water, for example, by *Procerodes* extracts (cf. Table VIII). Even if one assumes that the collodion membranes used were freely permeable to calcium, the calcium content could be reduced by more extreme dialysis than that used and still leave enough calcium to be definitely protective as compared with a similarly hypotonic sea water control.

The demonstration that the protective action of these biologically conditioned solutions is due to the increased calcium content in so far as the resistance of *Procerodes wheatlandi* to hypotonic water is concerned, brings this phenomenon into line with the greater resistance shown by *Procerodes* (= *Gunda*) *ulvæ* to fresh water when the calcium content is high. This is hardly the place, nor is *Procerodes* necessarily the most favorable material for an inquiry into the mechanism of calcium protection under these conditions.

The analyses of Pantin and his associates and of Beadle (1931, 1934) indicate that *P. ulvæ* lives in osmotic equilibrium with sea water and reaches a steady physiological state in certain fresh waters. This steady state is not the result of simple osmotic balance but is dependent largely on the calcium content of the water. It is maintained in part by a change in the permeability of the surface epithelium and is accompanied by increased respiration; it cannot be maintained indefinitely in extremely hypotonic waters. It seems highly probable (cf. Weil and Pantin, 1931) that the mechanism with these planarians is related to that in other cells in certain of which it has been shown, *Arbacia* eggs for example (McCutcheon and Lucke, 1928), that calcium decreases cell permeability to water and (Heilbrunn, 1930) favors membrane formation at a broken surface. The protection in these cases appears to be due to a decreased water intake rather than to a decreased leaching out of materials essential for continued existence.

We have not investigated the range of concentrations of calcium which would delay cytolysis in *Procerodes* placed in hypotonic water; in fact, we have established neither the upper nor the lower effective concentration. We know that an increase in specific conductance produced by the addition of calcium chloride equal to 1.08×10^{-5} mhos will give measurable protection. A rough calculation shows that this is approximately equivalent to the introduction of M/2600 $CaCl_2$. This approaches the lower limit of effectiveness under the conditions of our experiments. Buchanan (1935) reports that complete cytolysis readily occurs in *Euplanaria dorotocephala* in distilled water while in distilled water solutions of $CaCl_2$ there is no cytolysis in concentrations from M/500 to M/40,000 and cytolysis is distinctly delayed in a dilution of M/100,000. He found a detectable protective action in a M/1,000,000 solution.

We have been engaged in investigating the relation between calcium and the protective action of biological conditioning in extremely hypotonic waters when the calcium content is relatively low. We have little evidence regarding the effectiveness or non-effectiveness of such conditioning when the calcium is high. There are some indications in the first three significant lines in Table VI where full strength synthetic river water was used, that such conditioning may be effective. If so, some other mechanism than an increase in calcium is probably acting. These conditions approximate those found when a hard water stream from a limestone region flows into the ocean (cf. Breder, 1934). There is evolutionary and ecological as well as physiological interest in the relations between numbers present and the effectiveness of the invasion of such waters. Our present work does not deal with this question. However, it does show that marine invasions by animals with the physiological requirements of *Procerodes* into such relatively soft fresh waters as we have investigated is definitely favored by the presence in these waters of calcium excretors such as *Euplanaria novanglia* has been shown to be.

We have no information concerning the actual importance of such conditioning under natural conditions.

Throughout these studies there has been evidence of two opposing effects of numbers upon the resistance of these worms to fresh water: (*a*) the protective effect of freshly conditioned water or of numbers of animals present and (*b*) the harmful effect of numbers which shows up in conditioned media that have been allowed to go stale. At the osmotic level of these experiments, the protective effect is due to an increase in calcium and while the harmful effect has not been analyzed, it is a good *a priori* guess that the observed ill effects are associated with the accumulation of waste products of metabolism or to decomposition products of these.

The series of experiments of which this is the fourth report, were begun and have been prosecuted primarily in an investigation of a phase of mass physiology associated with animal aggregations (cf. Allee, 1931, 1934). The original impetus towards these particular experiments came from the observations of Drzewina and Bohn (1920, 1928) that the marine turbellarian *Convoluta roscoffensis* survives longer in hypotonic water if present in numbers than if isolated. This protection they attributed to the more rapid production of some sort of autoprotective substance by the group than would be possible for a single individual. This suggestion is now seen to have been essentially correct and, under the conditions of our experiments, to be composed of nothing more mysterious than calcium.

SUMMARY

1. An increase in the calcium content of extremely hypotonic water, when the calcium content is less than that found in sea water, delays the onset of fatal cytolysis for *Procerodes wheatlandi* isolated in such media.

2. Other electrolytes, even when increased appreciably, do not show this same protection; however, some protection of an osmotic nature is apparent.

3. The increase of calcium in biologically conditioned fresh waters is adequate to explain their observed protection for *Procerodes*.

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